CYTOMEGALOVIRUS IN THE PROCESS OF CHRONIC ALLOGRAFT NEPHROPATHY

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Academic dissertation

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LIST OF ORIGINAL PUBLICATIONS

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I Helanterä I, Koskinen P, Törnroth T, Loginov R, Grönhagen-Riska C, Lautenschlager I. The Impact of Cytomegalovirus Infections and Acute Rejection Episodes on the Development of Vascular Changes in 6-Month Protocol Biopsy specimens of Cadaveric Kidney Allograft Recipients. Transplantation 2003; 75 (11): 1858-1864.

II Helanterä I, Teppo A-M, Koskinen P, Törnroth T, Grönhagen- Riska C, Lautenschlager I. Increased Urinary Excretion of Transforming Growth Factor- β_1 in Renal Transplant Recipients During Cytomegalovirus Infection. Transplant Immunology 2006; 15: 217-221.

III Helanterä I, Loginov R, Koskinen P, Törnroth T, Grönhagen- Riska C, Lautenschlager I. Persistent cytomegalovirus infection is associated with increased expression of TGF-beta1, PDGF-AA and ICAM-1 and arterial intimal thickening in kidney allografts. Nephrology Dialysis Transplantation 2005 Apr; 20(4): 790-796.

IV Helanterä I, Koskinen P, Finne P, Loginov R, Kyllönen L, Salmela K, Grönhagen-Riska C, Lautenschlager I. Persistent Cytomegalovirus Infection in Kidney Allografts Is Associated with Inferior Graft Function and Survival. Submitted.

ABBREVIATIONS

CAN	Chronic allograft nephropathy
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
CRAD	Chronic renal allograft dysfunction
CsA	Cyclosporine A
CTGF	Connective tissue growth factor
EIA	Enzyme immunoassay
ELAM-1	Endothelial leucocyte adhesion molecule-1
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
HLA	Human leucocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IL	Interleukin
LFA-1	Lymphocyte function associated antigen
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa B
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
RAS	Renin-angiotensin system
RIA	Radio immunoassay
sLeX	Sialyl Lewis X
TGF-β	Transforming growth factor- beta
TNF- α	Tumor necrosis factor-alfa
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

ABSTRACT

Cytomegalovirus (CMV) is a major cause of morbidity, costs and even mortality in organ transplant recipients. CMV may also enhance the development of chronic allograft nephropathy (CAN), which is the most important cause of graft loss after kidney transplantation. The evidence for the role of CMV in chronic allograft nephropathy is somewhat limited, and controversial results have also been reported.

The aim of this study was to investigate the role of CMV in the development of CAN, and also to study the pathogenetic mechanisms behind this suggested association. Material for the purpose of this study was available from altogether 70 kidney transplant recipients who received a kidney transplant between the years 1992-2000. CMV infection was diagnosed with pp65 antigenemia test or by viral culture from blood, urine, or both. The presence of CMV in the kidney allograft biopsies was demonstrated by immunohistochemical detection of CMV pp65 protein, or by DNA hybridization in situ. Cytokines, adhesion molecules, and growth factors were demonstrated from allograft biopsies by immunohistochemistry, and from urinary samples by ELISA-methods. CMV infection and the presence of CMV in the allograft was correlated to histological changes in the 6-month protocol biopsies, levels of adhesion molecules and growth factors, and to long-term allograft function and survival during the five year follow-up.

CMV proteins were detectable in the 6-month protocol biopsies from 18/41 recipients with evidence of CMV infection. In the histopathological analysis of the 6-month protocol biopsies, presence of CMV in the allograft together with a previous history of acute rejection episodes was associated with increased arteriosclerotic changes in small arterioles, compared to recipients with no evidence of CMV infection after transplantation.

In urinary samples collected during CMV infection, excretion of TGF-β was significantly increased compared to recipients with no evidence of CMV infection. In recipients with increased urinary excretion of TGF-β, increased interstitial fibrosis was recorded in the 6-month protocol biopsies.

In biopsies taken after an active CMV infection, CMV persisted in the kidney allograft in 17/48 recipients, as CMV DNA or antigens were detected in the biopsies more than 2 months after the last positive finding in blood or urine. This persistence was associated with increased expression of TGF-β, PDGF, and ICAM-1 and with increased vascular changes in the allografts. Graft survival and graft function one and two years after

transplantation, as measured by estimated glomerular filtration rate, was reduced in recipients with persistent intragraft CMV. Persistent intragraft CMV infection was also a risk factor for reduced graft survival in Cox regression analysis, and an independent risk factor for poor graft function one and two years after transplantation in logistic regression analysis. No differences were recorded in the number of CMV seronegative recipients receiving an organ from a CMV seropositive donor, or in the number of acute rejection episodes between the study groups.

In conclusion, these results show that persistent intragraft CMV infection is detrimental to kidney allografts, causing increased expression of growth factors and increased vascular changes, leading to reduced graft function and survival. One possible link to this association could be the increased expression of the profibrotic and vasculopathic molecule TGF-β, observed in our study during both acute and persistent CMV infections.

REVIEW OF THE LITERATURE

1. KIDNEY TRANSPLANTATION

The first successful kidney transplantation was performed in 1954 between identical twins in Boston, USA [Murray et al. 1958]. Since the introduction of immunosuppressive drugs, and especially cyclosporine in the late 1970s [Kahan et al. 1985], kidney transplantation has become the standard curative treatment for end-stage renal disease. It is cost-effective, improves quality of life and is highly beneficial for patient survival compared to maintenance dialysis therapy [Wolfe et al. 1999, Cameron et al. 2000, Winkelmayer et al. 2002]. Since 1964, almost 5000 kidneys have been transplanted in Finland, and approximately 150-190 kidney transplantations are performed annually [Salmela and Kyllonen 2004]. The most important primary renal disease causing kidney transplantation in Finland is diabetic nephropathy, which is constantly increasing in number. Other major causes for transplantation in Finland include various glomerulonephritises and polycystic kidney disease. Worldwide, the most important cause of renal transplantation is glomerular diseases, followed by diabetic nephropathy [U.S. Department of Health and Human Services 2005, ERA-EDTA Registry 2005].

The results of kidney transplantation have been improving significantly over the past decades. According to the Finnish transplant registry data, the overall 1-year graft survival is nowadays approximately 93%, and graft half-life is more than 20 years [Salmela and Kyllonen 2004] (Figure 1), whereas in the United States, 1- year graft survival in cadaveric kidneys is approximately 89 % and graft half-life more than 10 years [U.S. Renal Data System 2005]. To prevent rejection, patients have to receive life-long immunosuppressive medication after transplantation. Triple- drug immunosuppression with cyclosporine, mycophenolate mofetil and methylpredisolone is the standard regimen in Finland nowadays, with high immunological risk patients receiving tacrolimus instead of cyclosporine.

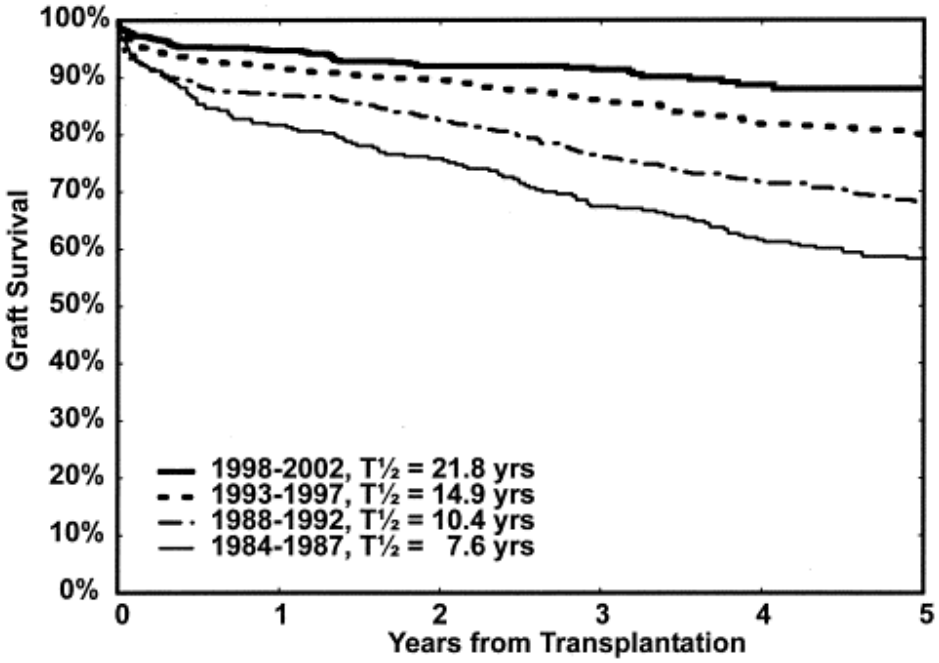

Figure 1. *Graft survival and graft half-life estimates for 1-year survivors from different time periods in Helsinki (N=2445). Reprinted from Salmela and Kyllönen 2004, with permission from the authors and the publisher (Elsevier).*

Important complications early after transplantation include hyperacute rejection, delayed graft function or primary nonfunction, renal arterial or vein thrombosis, and urinary tract complications. After the first weeks post transplantation, the most important complications are rejection and infections. Acute rejection develops in approximately 20% of kidney transplant recipients in Finland [Salmela and Kyllönen 2004] and is mostly reversible when treated with high-dose intravenous corticosteroids, or the administration of OKT3. The most important pathogens in immunocompromised organ transplant recipients are herpesviruses, especially cytomegalovirus (CMV), other viruses, and pneumocystis carinii.

Although the short-term results of kidney transplantation have improved greatly during the past decades, the long- term results have not improved accordingly [Meier-Kriesche et al. 2004]. The most important threats to long-term allograft success are death from vascular, malignant or infectious cause, and loss of allograft from chronic allograft dysfunction, often histologically defined as chronic allograft nephropathy [Chapman et al. 2005]. The third common cause of renal allograft loss, after death with a functioning graft and chronic allograft nephropathy, is recurrent glomerulonephritis in the allograft [Briganti et al. 2002].

2. CHRONIC ALLOGRAFT NEPHROPATHY

2.1. Terminology

The term “chronic allograft nephropathy” (CAN) was introduced in the 1990s to replace the term “chronic rejection”, since also non-alloimmune risk factors contribute to this process of allograft deterioration [Solez et al. 1993]. “Chronic rejection” applies only to the alloantigen-dependent mechanisms of chronic changes in the graft, and should be limited only to describe true alloimmune injury [Racusen et al. 1999]. The term “Chronic renal allograft dysfunction” (CRAD) is commonly also used to describe this same process but it is not a synonym of CAN. Chronic allograft nehropathy can be diagnosed only by kidney transplant biopsies and is the histopathological picture seen in transplanted kidneys. CAN represents the common pathway for renal allograft damage and is often associated with allograft dysfunction. Histological changes, however, are often seen in protocol biopsies before deterioration of allograft function; CRAD is thus always associated with CAN, whereas CAN may precede the development of CRAD [Kreis and Ponticelli 2001]. Chronic renal allograft dysfunction is therefore the functional consequence of chronic allograft nephropathy.

The use of CAN to describe all the processes leading to allograft dysfunction has also been criticized, since it may inhibit the goal to a specific diagnosis and appropriate therapy, and “has little value other than hide our ignorance” [Colvin 2003].

2.2. Clinical and histological findings

The pathogenesis of CAN is incompletely understood, but it is thought to result from various injuries to the allograft [Racusen et al. 1999] and several risk factors have been identified. There is no effective treatment currently available for CAN, mostly because histological signs of CAN represent late and irreversible damage. CAN ultimately leads to chronic renal allograft dysfunction and graft loss; together with death with a functioning graft, it is the most important reason for graft loss in the long-term, representing a major limitation to kidney transplant long-term success [Paul 1999, Chapman et al. 2005].

The earliest signs of CAN can be seen already after the first months post- transplantation [Seron et al. 2002], and evidence reports the prevalence of histological changes attributable to CAN being between 60- 70 % two years post transplantation [Solez et al. 1998]. In a study by Nankivell et al, all biopsies showed features of CAN five years after transplantation, and at 10 years severe CAN was present in nearly 60% of grafts

[Nankivell et al. 2003]. Clinically CAN is characterized by a slow rate of decline in kidney function, also hypertension and proteinuria may be present [Paul 1999].

The international Banff scheme and classification provide tools for the scoring of histopathological changes attributable to both acute rejection and chronic changes [Solez et al. 1993, Racusen et al. 1999, Racusen et al. 2003]. The most important histopathological findings in CAN are interstitial fibrosis and tubular atrophy [Racusen et al. 1999, Chapman et al. 2005]. Vascular findings in CAN include fibrointimal thickening in arteries and arterioles and arteriolar hyalinosis. The Banff scheme also describes specific vascular findings caused by true chronic rejection by alloimmune mechanisms: disruptions of the elastica, inflammatory cells in the vascular intima, proliferation of myofibroblasts in the intima and the formation of neointima. Glomerular changes in CAN are described as double contours in capillary loops, glomerular mesangial matrix increase and ultimately glomerular sclerosis. The natural history of CAN has been described recently [Nankivell et al. 2003]. During the first year after transplantation, the histological picture is characterized by rapidly increasing interstitial fibrosis and tubular atrophy, which are also the histopathological changes least sensitive to sampling errors; later damage presents as increasing vascular and glomerular changes, and also further interstitial fibrosis.

2.2. Risk factors

2.2.1. Acute rejections

The most important risk factor for the development of CAN is thought to be acute rejection episodes [Almond et al. 1993, Lindholm et al. 1993, Meier-Kriesche et al. 2000]. According to the Banff ’97 classification, acute cellular rejection is histologically classified as borderline (mild tubulitis and mild interstitial inflammation), tubulointerstitial rejection without arteritis (type Ia moderate and type Ib severe), vascular rejection (type IIa mild-to-moderate intimal arteritis, type IIb severe intimal arteritis) and severe rejection (type III transmural arteritis and fibrinoid or smooth muscle necrosis) [Racusen et al. 1999]. Recently, antibody mediated rejection was added to the Banff scheme, identified as positive C4d deposits in peritubular capillaries or immunoglobulin and complement in fibrinoid necrosis, together with serologic evidence of circulating antibodies to donor HLA [Racusen et al. 2003].

Risk of CAN increases with the number of acute rejection episodes, and the intensity and type of rejection have also been shown to be important [Matas et al. 1994]. Vascular rejection is a more serious form of rejection and is more harmful to the graft [van Saase et al. 1995], and acute antibody mediated rejection has been associated with poor

prognosis compared to acute cellular rejection [Herzenberg et al. 2002]. Furthermore, late acute rejections occurring months or years after transplantation are associated with a greater risk of graft loss compared to early acute rejection episodes [Sijpkens et al. 2003].

2.2.2. Other immunological risk factors

Increased number of mismatched MHC antigens is strongly associated with inferior graft survival [Held et al. 1994, Opelz et al. 1999]. The exact independent role of HLA-mismatches is, however, somewhat questionable, since the logistics of HLA-matching and organ allocation in areas of long geographic distances increase cold ischemia times [Schnitzler et al. 1999]. Although contradictory views have also been expressed [Starzl et al. 1995, Terasaki et al. 1995], HLA- matching is still regarded as important in increasing the long-term prognosis of kidney allografts [Opelz et al. 1999, Opelz 2001]. Also previous sensitization and the presence of preformed antibodies at the time of transplantation, or development of HLA antibodies after transplantation, present a risk for CAN [Terasaki 2003, Terasaki and Ozawa 2004, Hourmant et al. 2005].

2.2.3. Non- immunological risk factors

Delayed graft function is usually defined as the need for postoperative dialysis during the first week after transplantation. It is thought to result from ischemia-reperfusion injury, which triggers an inflammatory response, leading to increased immunogenicity and the risk of early acute rejection [Perico et al. 2004]. The terms acute tubular necrosis and delayed graft function are often used to define the same disorder, although they are not synonyms: acute tubular necrosis is a histopathological diagnosis and can also occur in a functioning graft, whereas delayed graft function is a clinical diagnosis. Delayed graft function is thought to increase the risk of CAN [Shoskes and Cecka 1998, Perico et al. 2004]. Increased cold ischemia time is associated with a risk of DGF and acute rejection episodes, and also with the development of CAN [Shoskes and Cecka 1998]. Whether fully recovered delayed graft function in the absence of acute rejection episodes has any effect on long-term allograft survival, is still a controversial issue, with evidence existing in support of both hypotheses [Troppmann et al. 1996, Shoskes and Cecka 1998].

Increased donor age has a strong negative effect on graft function and survival after transplantation [Terasaki et al. 1997]. Kidneys from older donors have suffered more damage and have a smaller number of functional nephrons, and may have a reduced capacity to repair damage adequately [Halloran et al. 1999]. Furthermore, many of the histological changes associated with CAN are also seen in the aging kidney, such as arteriolar hyalinosis, interstitial fibrosis, tubular atrophy, and glomerular sclerosis. Older donor age increases the immunogenicity of the graft, resulting in more acute cellular rejection episodes [de Fijter et al. 2001]. In addition, acute rejection episodes in old

kidneys have a greater risk of progressing to graft loss [de Fijter et al. 2001]. Also very young donor age is associated with a poorer prognosis after transplantation [Neumayer et al. 1994]. Donor source is also a major contributor to long-term graft survival, since grafts from living donors have a significantly longer half-life than cadaveric grafts from brain-dead donors [Terasaki et al. 1995, Hariharan et al. 2000], although this may apply only in the presence of acute rejection episodes [Knight et al. 2001].

Several classical risk factors for atherosclerosis may increase the risk of CAN, such as hypercholesterolemia [Dimeny et al. 1995, Roodnat et al. 2000], hypertension [Opelz et al. 1998], diabetes [Miles et al. 1998] and smoking [Sung et al. 2001]. Reduced renal function at baseline and also decline of function during the first year are important determinants of long- term graft survival, although that may only represent a previous injury [Kasiske et al. 2002]. In epidemiological studies, several other risk factors for CAN have also been identified, such as hypoalbuminemia, recipient young age, and recipient black race [Massy et al. 1996, Young and Gaston 2000]. Infections caused by cytomegalovirus (CMV) have also been associated with decreased graft survival [Nett et al. 2004], although controversial evidence exists as well [Dickenmann et al. 2001].

3. OTHER CAUSES OF ALLOGRAFT DYSFUNCTION

3.1. Calcineurin inhibitor nephrotoxicity

After the introduction of cycloposporine (CsA) in the 1970s, calcineurin inhibitors (CNI) have been the backbone of immunosuppression in kidney transplantation, enabling also the transplantation of other solid organs. The immunosuppressive effect of CNI:s cyclosporine and tacrolimus is based on the inhibition of the phosphatase calcineurin, which plays a key role in T-cell activation [Kahan 1989]. Although the immunosuppressive potential of CNI:s has resulted in improved early graft survival and reduced acute rejection rates, a major concern for decades has been the nephrotoxicity of both CsA and tacrolimus, although most of the research data is from cyclosporine [Calne et al. 1978, Myers et al. 1984, Bennett et al. 1996, Finn 1999, Nankivell et al. 2004]. CsA nephrotoxicity can be roughly divided into an acute form, which is reversible by reducing the dose of CsA, and chronic nephrotoxicity, which is somewhat poorly documented but is thought to be irreversible [Bennett et al. 1996, Burdmann et al. 2003]. Calcineurin inhibitors cause injury to the kidneys by several mechanisms, such as renal vasoconstriction and upregulation of fibrotic growth factors [Bennett et al. 1996, Khanna et al. 2002, Burdmann et al. 2003]. Clinically CsA nephrotoxicity presents as impaired renal function, electrolyte disturbances and hypertension. Histopathological characteristics suggested to be specific for CsA nephrotoxicity include nodular arteriolar hyalinosis, tubular microcalcification, isometric tubular vacualization, striped cortical

fibrosis, peritubular and capillary congestion and juxtaglomerular hyperplasia [Bennett et al. 1996]. The contribution of CsA nephrotoxicity to CAN is very difficult to estimate. By definition, obvious CsA nephrotoxicity with specific histopathological characteristics should be excluded from CAN [Halloran et al. 1999]. Some chronic lesions of CAN and CsA nephrotoxicity, such as arteriolar hyalinosis and interstitial fibrosis, however, are indistinguishable [Colvin 2003]. Whether CsA nephrotoxicity is a key player in the progression of CAN, or whether adequate doses of CNIs protect against CAN remains one of the major unknown issues in kidney transplantation [Halloran et al. 1999].

3.2. Infections

Infections are also able to cause allograft dysfunction. Bacterial urinary tract infections after kidney transplantation are common and are able to cause graft dysfunction, but they have also been associated with acute rejections [Tolkoff-Rubin and Rubin 1992, Yang et al. 1994, Nampoory et al. 2003], although controversial evidence exists as well [Maki et al. 1992]. Also other bacterial infections have been reported to be associated with graft dysfunction, such as mycobacterial infections, respiratory tract infections and septicemia [Schmaldienst and Horl 1997, Nampoory et al. 2003, Queipo et al. 2003, Moroni et al. 2004].

The most common viral infection after renal transplantation is caused by cytomegalovirus (CMV) [Patel and Paya 1998]. CMV is able to cause graft dysfunction during acute infection, and may be associated with long-term complications. The effects of CMV on kidney transplants are described in detail below. Chronic kidney transplant dysfunction can also be caused by polyomavirus hominis 1 virus, better known as BK virus (BKV), infecting up to 90% of the general population. BKV replication is seen in 10-60% of recipients after kidney transplantation, and BKV can cause ureteric stenosis, transient graft dysfunction, or irreversible graft failure [Hirsch and Steiger 2003]. Evidence indicates that polyomavirus associated nephropathy is seen in 1-10% of kidney allograft recipients, and it leads to graft failure in 10-80% of cases [Hirsch et al. 2005]. Closely related to BK virus is polyomavirus hominis 2 virus, JC virus, which also infects renal transplants [Hirsch et al. 2005]. The clinical significance of JCV in transplant dysfunction remains to be resolved.

4. PATHOGENESIS OF CAN

The pathogenesis of chronic allograft nephropathy is still not fully elucidated, although several theories have been suggested [Hayry et al. 1993, Halloran et al. 1999, Paul 1999, Joosten et al. 2004]. Chronic allograft nephropathy is thought to originate from a series of challenges to the allograft. Injury to the graft begins even before the effect of the alloresponse: donor brain death, warm ischemia, cold ischemia, and ischemia-

reperfusion injury all result in increased immunogenicity in the graft, causing increased inflammatory alloresponse after the revascularization of the allograft. Series of injuries continues during the first weeks after transplantation; acute tubular necrosis, acute rejection episodes, cyclosporine nephrotoxicity, and infections in the graft, among others, contribute to the injury of the transplanted kidney. All this occurs against the background of foreign MHC antigens and often in a kidney from an older donor with some extent of age-associated changes and limited capacity of restoration from injury. Several mechanisms have been suggested for the development of CAN. Alloimmune mechanisms are thought to be central in the development of CAN. Renal injury is thought to result in an inflammatory response; recipient lymphocytes and monocytes enter the graft and produce cytokines, which stimulate inflammatory and mesenchymal cells to produce excess growth factors, resulting in proliferation of myofibroblasts and smooth muscle cells in the vascular wall and increased of collagen synthesis in fibroblasts. This process is thought to lead to scar formation; excess interstitial fibrosis, tubular atrophy and vascular intimal thickening represent the stereotypic histopathological picture seen in end-stage renal diseases. Whether the initial injury in the graft occurs in the vascular wall, resulting in the proliferation of the vascular wall and narrowing of the lumen, followed by tubulointerstitial lesions due to growth factor response and partly ischemia [Hayry et al. 1993], or whether the initial event is tubular cell injury, resulting in growth factor response, tubular atrophy, interstitial fibrosis, and finally vascular narrowing, is still under discussion [Paul 1999]. Recent description of the histopathological natural history of CAN supports the latter hypothesis [Nankivell et al. 2003]. In their study, interstitial fibrosis and tubular atrophy were the first manifestations of CAN and preceded vasculopathic changes.

Most of the histopathological changes of CAN are also seen in the aging kidney. This fact and knowledge of the limited cell cycle capacity has led to the theory that cellular or tissue senescence might play a role in the pathogenesis of CAN [Halloran et al. 1999]. Continuous injury to an aging graft may result in exhaustion of the replicative cells and inability to repair injury and remodel the tissue in an adequate way. This is thought to lead to the histopathological lesions seen in CAN.

On the other hand, glomerular hyperfiltration due to decreased renal mass and smaller number of functional nephrons after injuries to the graft, or perhaps also from severe donor-recipient size mismatch, may contribute to the development of CAN [Paul 1999]. Severe persistent glomerular hypertension may lead to changes in glomerular permeability and leakage of molecules, resulting in an increase in the glomerular mesangium, ultimately leading to glomerulosclerosis. Changes in glomerular permeability also may lead to leakage of proteins to the urine. Proteinuria is thought to

have a causative role in the development of tubulointerstitial damage, either by direct tubular cell injury caused by albumin, or by a more complex process of growth factors in the glomerular ultrafiltrate, stimulating tubular cells and causing interstitial fibrosis [Hirschberg and Wang 2005]. Increasing evidence from the pathogenesis of renal fibrosis in several primary fibrotic renal diseases highlights the importance of tubular cells as the pivotal cells in renal fibrosis; tubular cells possess important secretory properties and in fibrosis, increased number of fibroblasts derives from tubular cells by epithelial-mesenchymal transition, also after kidney transplantation [Iwano and Neilson 2004, Vongwiwatana et al. 2005]. Fibrosis is thought to represent a final physiological pathway of renal damage regardless of the underlying primary cause [Chatziantoniou and Dussaule 2005], and this could also apply to kidney transplant pathophysiology.

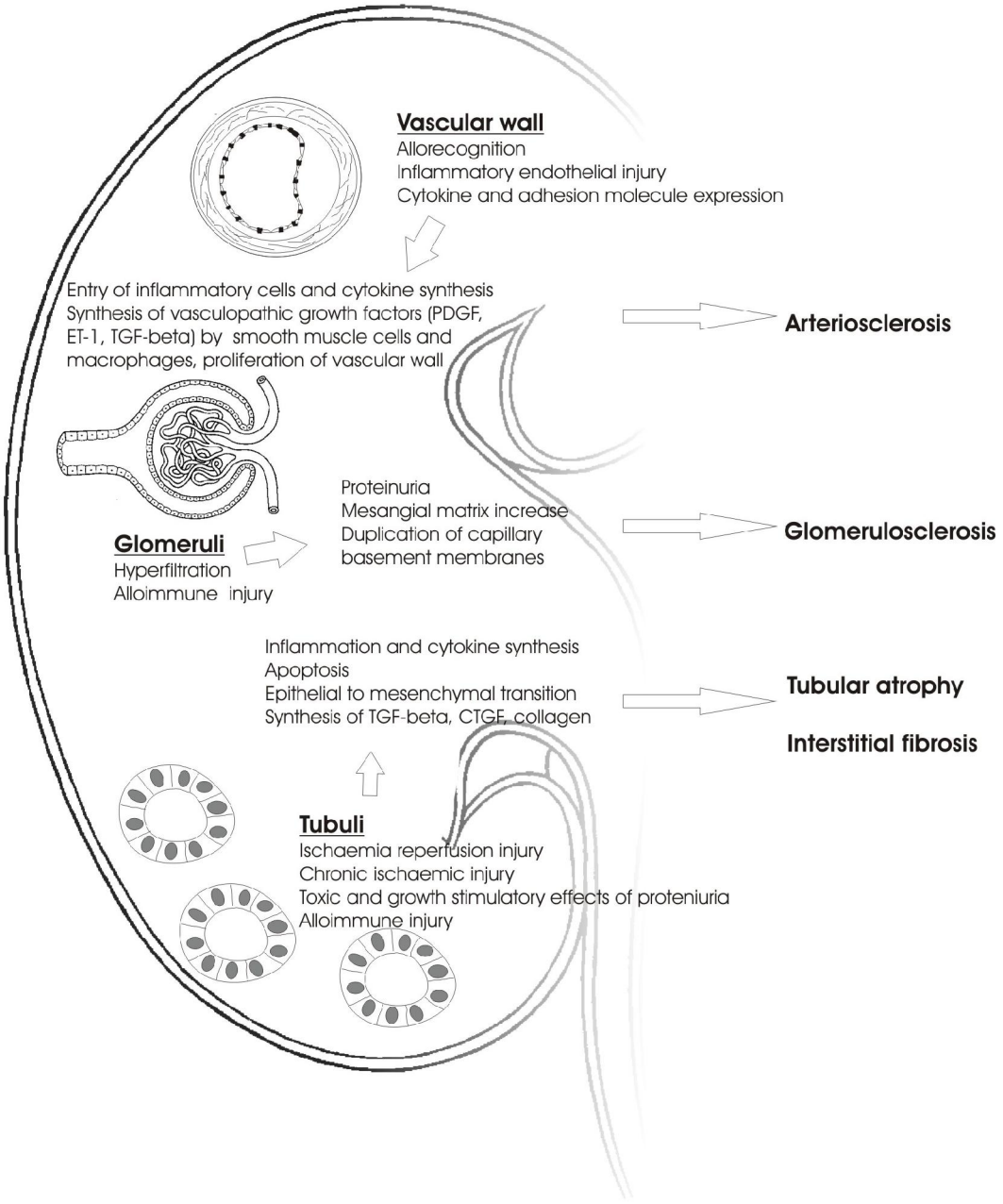

Figure 2. *Suggested mechanisms of the pathogenesis of chronic allograft nephropathy*

4.1. Alloresponse

The process of alloresponse begins in allorecognition, which can occur in two major ways, by direct and indirect recognition. In the direct allorecognition pathway, recipient T cells recognize intact foreign MHC antigens on the surface of donor-derived antigen presenting cells that are also able to provide the necessary co-stimulation for T cell activation. This direct pathway results mainly in cytotoxic CD8+ T cell response and plays a role in early allograft rejection [Sayegh 1999]. In indirect allorecognition, recipient-derived antigen presenting cells present fragments of donor-derived foreign MHC-peptides, which they have phagocytized and processed to their MHC class II surface molecule peptide-binding clefts, to recipient T cells. In the presence of the necessary co-stimulatory molecules and cytokine milieu, T cells become activated. The indirect pathway activates mostly CD4+ helper T cells. Evidence shows that the indirect allorecognition pathway enhances acute rejection and is important in chronic alloimmune response [Sayegh 1999].

Cytokines are secreted by different cell types, such as monocytes and T cells, especially CD4+ T cells. Cytokines stimulate target cells by binding to their specific receptors on the target cell surface and they can stimulate cells in an autocrine, paracrine, or endocrine fashion. Proinflammatory cytokines increase inflammatory reaction by stimulating inflammatory cells, such as T cells, B cells, and macrophages. They also increase MHC expression on cell surfaces, stimulate the expression of adhesion molecules on epithelial and endothelial cell surfaces, and also influence the synthesis of other cytokines, some of which are also inhibitory to the process. The type of cytokine response determines the type and intensity of immune response.

In the beginning of the immune response, proinflammatory cytokines, such as interferon-gamma (IFN- γ), interleukins -1 and -2 (IL-1 and IL-2) and tumor necrosis factor, direct the immune response towards cell-mediated immune response (Th1). Th1 cells secrete IL-2 and IFN-γ, which stimulate cytotoxic and delayed-type hypersensitivity response. In contrast, Th2 cells secrete cytokines such as IL-4, IL-5, IL-6, and IL-10, which direct the immune response towards antibody mediated immune response and inhibit cell-mediated immune response [Dallman 2001]. Both cell-mediated immune response and humoral response are thought to be important in the development of chronic changes in the graft [Sayegh 1999, Terasaki 2003].

The role of alloimmune response is thought to be central in the development in CAN [Joosten et al. 2004]. Although several non-immunological risk factors for CAN have been described above, some of them, such as increased donor age and ischemia-reperfusion injury, result in increased immunogenicity of the allograft, and the injury to

the graft is thought to be mainly alloimmune mediated. The division between alloantigen-dependent and independent risk factors is therefore somewhat arbitrary.

Adhesion molecules play a key role in the early phase of the alloimmune response. They are mainly expressed on the surface of epithelial and endothelial cells, but also on antigen presenting cells [Solez et al. 1997]. Entry of the inflammatory cells from the bloodstream to the allograft is a complex process mediated by cytokines, chemotactic factors and adhesion molecules. After rolling of leucocytes along the vessel wall, firm adhesion of leucocytes to the vascular wall at the site of inflammation, and also transmigration of leucocytes into the tissue, is mediated by adhesion molecules [Springer 1994]. Adhesion molecules are also involved in T cell activation [Altmann et al. 1989, Lebedeva et al. 2005].

Expression of adhesion molecules is stimulated by proinflammatory cytokines, such as tumor necrosis factor-alfa (TNF-α), IL-1 and IFN-γ, during the initiation of an inflammatory process. The most important adhesion molecules in the context of allograft rejection are thought to be intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leucocyte adhesion molecule (ELAM-1) and their ligands lymphocyte function associated antigen-1 (LFA-1), very late antigen-4 (VLA-4), and Sialyl Lewis X (sLeX) expressed on leucocytes [Solez et al. 1997, Fuggle and Koo 1998]. The expression of these adhesion molecules is increased during acute renal allograft rejection in tubular, glomerular, and endothelial cells. Increased expression of ICAM-1 and VCAM-1 has also been seen in chronic renal allograft rejection [Hill et al. 1995]. Treatment with antibodies against adhesion molecules can reverse or prevent acute rejection in animal models [Cosimi et al. 1990], although a large clinical trial with anti-ICAM-1 monoclonal antibody showed no benefit after kidney transplantation [Salmela et al. 1999], suggesting that actions of ICAM-1 can be overcome by other components of the immune system.

4.2. Growth Factors

Proinflammatory cytokines produced by the inflammatory alloresponse stimulate several cell types, both inflammatory and mesenchymal cells, to produce different growth factors that are thought to lead to a repair process in response to the injury, and contribute to the chronic changes seen in the graft. Several growth factors have been identified to be important in the process of CAN.

4.2.1. Transforming growth factor-β (TGF-β)

Transforming growth factor-β is a multifunctional cytokine thought to play a central role in tissue fibrosis in different organs and different pathogenic states [Border and Noble

1994]. TGF-β exists in three different isoforms, whose biological properties are very similar. Most of the current data arise from TGF-β_1, which is the most abundant isoform in all tissues. It is secreted by platelets and various inflammatory cells in response to injury, as an inactive precursor bound to a latency-associated peptide, and it requires activation before exerting its biological effects. Expression of TGF-β is induced by cytokines, such as IL-1 and TNF-α, and also by autoinduction [Van Obberghen-Schilling et al. 1988, Kim et al. 1990]. TGF-β directly stimulates the synthesis of several extracellular matrix components, modulates the composition of the matrix, and inhibits the degradation of extracellular matrix by blocking the secretion and functions of proteases. Some of the growth stimulatory actions of TGF-β are executed via indirect mechanisms, such as mesangial cell growth via the stimulation of platelet-derived growth factor (PDGF), and the increased production of fibroblast growth (FGF) factor in endothelial cells [Haberstroh et al. 1993]. TGF-β also stimulates the production of connective tissue growth factor (CTGF) [Grotendorst 1997], which is thought to be a key profibrotic growth factor in the development of renal fibrosis [Ito et al. 1998]. In addition to its growth stimulatory and profibrotic properties, TGF-β is also an anti-inflammatory molecule, as it inhibits T and B cells and their production of several cytokines, such as IL-1, IL-2 and TNF-α [Border and Noble 1994].

TGF-β is thought to play a key role in different fibrotic kidney diseases, such as diabetic nephropathy, chronic glomerulonephritis, and hypertensive glomerular injury [Border and Noble 1997, Ziyadeh 2004]. Increased expression of TGF-β can be seen in these conditions in both renal biopsy specimens and urine [Yamamoto et al. 1996, Honkanen et al. 1997, Korpinen et al. 2000]. Interstitial fibrosis is a key feature of chronic allograft nephropathy, and a wealth of experimental and clinical evidence supports a role for TGF-β in the process of CAN [Shihab et al. 1995, Sharma et al. 1996, Shihab et al. 1996, Cuhaci et al. 1999, Nicholson et al. 1999]. Similarly, the histopathological changes seen in both cyclosporine and tacrolimus nephrotoxicity are thought to be associated with increased expression of TGF-β, which is directly caused by calcineurin inhibitors [Shehata et al. 1995, Shihab et al. 1997a, Shihab et al. 1997b, Khanna et al. 1999].

4.2.2. Platelet- derived growth factor (PDGF)

Platelet- derived growth factor exists as a disulfide- linked homo- or heterodimer of two polypeptide chains (A and B chain), and can form three different isoforms, PDGF-AA, AB and BB, which all are biologically active [Heldin and Westermark 1999]. Their biological actions are exerted by PDGF receptors α and β. PDGF β receptor binds only PDGF-B chains, while PDGF α receptor binds both A and B chains. PDGF-C and D have been recently identified, but their exact biological role is not fully elucidated [Floege et al. 2003, Li and Eriksson 2003]. PDGF is an important stimulatory growth factor for

connective tissue cells, stimulating the chemotaxis and proliferation of fibroblasts and smooth muscle cells. It also stimulates the chemotaxis of neutrophils and macrophages, and their production of several other growth factors. Furthermore, PDGF stimulates the production of several extracellular matrix proteins.

PDGF plays an important role in the embryogenesis of several tissues, such as kidney, lung, and heart [Heldin and Westermark 1999]. In the kidney, overactivity of PDGF has been associated with the pathogenesis of various glomerulonephritises characterized by mesangial cell proliferation [Abboud 1995], tubulointerstitial fibrosis [Tang et al. 1996], and also with kidney transplant rejection [Fellstrom et al. 1989, Alpers et al. 1996, Floege et al. 2003, Savikko et al. 2003]. Some extent of interplay exists between TGF-β and PDGF [Battegay et al. 1990] and at least a part of the effect of TGF-β on mesangial cells may be mediated by PDGF [Haberstroh et al. 1993].

4.2.3. Others

Also several other molecules have been suggested to be important in the development of CAN, of which the most important are described below.

Renin-angiotensin system (RAS) has a fundamental physiologic role in the regulation of blood pressure, intravascular volume and electrolyte balance. Activation of the RAS and generation of angiotensin II plays a role in the pathogenesis of hypertension and renal fibrosis [Matsusaka et al. 1996], and inhibition of RAS by angiotensin converting enzyme-inhibitors, or angiotensin II receptor blockers, is routine clinical practice in the prevention of the progression of several kidney diseases. In addition to its hemodynamic properties, angiotensin II is a growth factor, which upregulates adhesion molecules and cytokines and promotes tissue fibrosis [Wolf and Neilson 1993]. Angiotensin II increases the secretion of TGF-β and a part of its profibrotic effects are though to be mediated by TGF-β [Border and Noble 1998]. Experimental and limited clinical evidence suggest that blocking RAS in kidney transplant recipients may be beneficial in the long term in preventing CAN and cyclosporine nephrotoxicity [Shihab et al. 1997c, Campistol et al. 1999, Szabo et al. 2000, Lin et al. 2002, Artz et al. 2004], supporting a role for angiotensin II in the development of CAN.

The generation of pathological renal fibrosis is a complex process, in which also the inhibition of extracellular matrix degradation may be important [Rerolle et al. 2000]. Plasminogen activator inhibitor type-1 (PAI-1) inhibits extracellular matrix degradation by several mechanisms. One mechanism, by which TGF-β and angiotensin II promote the generation of fibrosis, is the induction of the synthesis of PAI-1 [Kanalas and Hopfer 1997, Rerolle et al. 2000]. Evidence has associated increased expression of PAI-1 with

several fibrotic kidney diseases [Rondeau et al. 1990, Yamamoto et al. 1996], and increased expression of PAI-1 has been documented also in chronic allograft nephropathy [Tang et al. 1998, Grandaliano et al. 2001, Lahlou et al. 2002].

Endothelin-1 (ET-1) is a peptide with potent vasoconstrictor actions and it is also a stimulating growth factor for smooth muscle cells [Schiffrin 2001]. ET-1 has been associated with the pathophysiology of various conditions and is also thought to be one of the key players in renal fibrosis [Chatziantoniou and Dussaule 2005]. Increased levels of ET-1 have been associated with chronic vasculopathy in kidney allografts and increased expression of ET-1 has been documented also in tubular cells in acute and chronic rejection of kidney transplants [Simonson et al. 1998, Chareandee et al. 2000]. In experimental models, ET-1- receptor antagonists have prevented the development of CAN [Orth et al. 1999], supporting a role also for ET-1 in the development of CAN.

5. CYTOMEGALOVIRUS (CMV)

Cytomegalovirus (CMV) is a member of the betaherpesvirus subfamily, belonging to the family of herpesviruses [Mocarski and Courcelle 2001]. Human cytomegalovirus is a large DNA- virus of 150-200 nm in diameter, consisting of a linear double-stranded DNA of approximately 230 kbp inside an icosahedral capsid, surrounded by tegument or matrix, enclosed in an envelope of lipid bilayer carrying viral glycoproteins. Monocytes, macrophages, lymphocytes, and hematopoietic precursor cells are the main cell types of CMV infection and replication. In addition, CMV is able to infect several tissues, including most of the parenchymal organs, salivary glands, eye, gastrointestinal and genitourinary tract, and is also able to replicate in several cell types, including epithelial cells, endothelial cells, fibroblasts, smooth muscle cells and macrophages [Pass 2001]. The cellular receptor of CMV remains yet to be fully elucidated but due to the wide range of cells CMV is able to infect, the receptor is thought to be widely distributed [Mocarski and Courcelle 2001]. One suggested candidate is epidermal growth factor receptor [Wang et al. 2003]; others include cellular integrins [Feire et al. 2004]. Primary infection of CMV occurs commonly in childhood or during the first two decades of life. Primary infection is usually asymptomatic in an immunocompetent host but may present as a mononucleosis-like syndrome, or rarely as hepatitis or pneumonia [Pass 2001]. During primary infection and reactivations, viral shedding can be detected in saliva, breast milk, semen, and other body excretions for weeks to months. The seroprevalence of CMV varies between 40 and 100% in different countries, being approximately 70-80% in Finland and Scandinavian countries, whereas in the United Kingdom the seroprevalence of CMV is only approximately 40% [Ho 1990]. As with other members of the herpesvirus family, primary CMV infection leads to a life-long latency. During latency, the main reservoir of CMV is thought to be blood leucocytes, mainly mononuclear cells, but also hematopoietic progenitor cells have been documented to carry latent viral DNA [Taylor-Wiedeman et al. 1991, Kondo et al. 1994, Sindre et al. 1996, Soderberg-Naucler et al. 1997]. During latency, CMV can be transmitted via blood products and transplanted organs. Cell-mediated immunity with both natural killer cells and cytotoxic T lymphocytes, is thought to be the most important defense mechanism to control CMV infection and humoral immunity may be of less importance [Harari et al. 2004]. In an immunocompromised host, either in individuals receiving immunosuppressive medication or in HIV infected individuals, CMV can activate from latency and can cause severe life-threatening infections.

5.1. CMV in Transplant Recipients

Cytomegalovirus is one of the most important pathogens in transplant recipients receiving immunosuppressive medication. Most of the CMV infections in these

individuals are caused by reactivation of latent virus of either recipient or donor origin. In transplant recipients with no previous history of CMV infection, primary infection occurs commonly and is often more severe compared to a reactivation of the virus in a seropositive host. CMV infection in organ transplant recipients manifests commonly as fever, malaise, leucopenia, or thrombocytopenia, but can also cause organ specific manifestations, such as hepatitis, gastrointestinal symptoms, renal dysfunction, and CMV pneumonitis, which still in the modern era of effective antiviral medication may be fatal. Diagnosis of CMV infection in organ transplant recipients is based on detection of the virus in blood, urine, biopsies, or bronchoalveolar lavage fluid. Shell vial cultures can be used to detect the virus in blood or urine [Gleaves et al. 1984] but a quantitative assay for the detection of CMV pp65 antigen in blood, developed in the late 1980s [van der Bij et al. 1988], is more sensitive and accurate [The et al. 1995]. Nowadays quantitative PCR to detect CMV DNA in the blood is the most common method to diagnose CMV infections, being less time-consuming and more specific [Pang et al. 2003, Boeckh et al. 2004, Piiparinen et al. 2004]. CMV proteins or genome can be demonstrated also in tissue sections of biopsy material. Serology has an important role prior to transplantation in evaluating the donor and recipient but after transplantation, serology has only a limited diagnostic value.

Symptomatic CMV infection occurs in 10-50% of organ transplant recipients, depending on the transplanted organ, and asymptomatic viral activation can be detected in the majority of recipients [Patel and Paya 1998]. CMV infection can be treated with a specific antiviral agent, the nucleoside analogue ganciclovir, which prevents viral replication but does not completely eliminate the virus. In case of ganciclovir treatment failure, or side effects, such as leucopenia, intravenous foscarnet may be used to treat CMV infections. In kidney transplant recipients, the use of foscarnet is restricted by its nephrotoxicity. Because of the high morbidity, costs, and even mortality associated with CMV, high-risk groups of seronegative recipients of an organ from a seropositive donor commonly receive prophylaxis with peroral valganciclovir, a valine ester of ganciclovir, during the first 3-6 months after transplantation. Another strategy is preemptive treatment, in which patients are frequently monitored and antiviral therapy is administered when evidence of CMV viremia is detected, before clinical symptoms develop. In addition to the symptomatic infection caused by CMV, several studies indicate that CMV enhances the development of chronic rejection in heart, lung, and liver transplantations [Grattan et al. 1989, Loebe et al. 1990, Arnold et al. 1992, Koskinen et al. 1993, Kroshus et al. 1997, Lautenschlager et al. 1997a, Evans et al. 1999, Valantine et al. 1999].

5.2. CMV in kidney transplantation

After kidney transplantation, approximately 10-20% of recipients suffer from symptomatic CMV infection [Patel and Paya 1998, Becker et al. 2002, Sagedal et al. 2002]. CMV seronegative recipients of an organ from a seropositive donor are at highest risk of symptomatic CMV infection. Asymptomatic activation of the virus can be detected in up to 60% of recipients [Sagedal et al. 2000]. Several studies have associated acute CMV infection with glomerulopathic changes [Richardson et al. 1981, Tuazon et al. 1987, Rao et al. 1994]. Evidence has also associated CMV with inferior graft survival [Fryd et al. 1980, Rubin et al. 1985, Lewis et al. 1988, Almond et al. 1993, Hirata et al. 1996] and with acute rejections [Simmons et al. 1970, von Willebrand et al. 1986, Pouteil-Noble et al. 1993, Reinke et al. 1994, Toupance et al. 2000, Sagedal et al. 2002]. Several recent studies have similarly associated CMV with chronic graft dysfunction and graft loss [Humar et al. 1999, Giral et al. 2001, Tong et al. 2002, Geddes et al. 2003, Sola et al. 2003, Nett et al. 2004, Sagedal et al. 2004], but contradictory data have also been reported [Akposso et al. 1997, Dickenmann et al. 2001, Ricart et al. 2005]. Table 1 summarizes the clinical evidence of CMV and chronic allograft nephropathy or chronic renal allograft dysfunction.

In addition to the suggested association between CMV and impaired graft survival, CMV has been associated with increased cardiovascular complications [Humar et al. 2000, Kalil et al. 2003], increased gastrointestinal complications [Kaplan et al. 1999, Sarkio et al. 2005], and with the development of post-transplant diabetes mellitus after kidney transplantation [Hjelmesaeth et al. 2004].

For CMV:	N	Description
Nett et al 2004	2740	In univariate analysis, CMV disease was a risk factor for graft loss (included also kidney-pancreas recipients, follow-up 2-10 years)
Humar et al 1999	1339	In multivariate analysis, CMV disease was a risk factor for biopsy proven chronic rejection only in the presence of previous acute rejection (biopsies taken only when chronic rejection suspected, follow-up 1-10 years)
Giral et al 2001	303	In a study comparing mycophenolate mofetil and azathioprine, CMV disease was associated with poorer graft survival but only in patients using azathioprine (follow-up 1-10 years)
Sagedal et al 2004	496	Asymptomatic CMV infection and CMV disease were risk factors for mortality and uncensored graft loss but not for death-censored graft loss (prospective study, weekly monitoring of CMV, follow-up 4-7 years)
Sola et al 2003	259	CMV disease but not asymptomatic CMV, was a risk factor for CRAD in multivariate analysis (weekly monitoring of CMV between days 30-90 post-transplantation, follow-up 0.5-4 years)
Geddes et al 2003	41	CMV infection and disease were associated with inferior renal function at one year post-transplantation
Tong et al 2002	37	CMV disease was associated with poor graft function at 6 months and with CAN (biopsies taken only when CAN was suspected, follow-up 3 years).
Against CMV:		
Akposso et al 1997	169	Preemptive therapy with ganciclovir was administered, when CMV viremia was detected. CMV infection had no impact on graft survival or renal function (follow-up 0-6 years)
Ricart et al 2005	205	In simultaneous kidney-pancreas recipients, CMV infection was associated with inferior renal function 1-3 months post-transplantation, but had no impact on graft survival (follow-up 3 years)
Dickenmann et al 2001	84	CMV infection was not associated with inferior graft function or survival (CMV monitoring every 1 to 2 weeks, follow-up 5 years)

Table 1. *The role of CMV in CAN during the era of modern immunosuppression: clinical evidence for and against CMV*

5.3. CMV and transplant rejection in experimental and molecular level

In experimental rat models of chronic kidney allograft rejection, CMV increases inflammation, enhances the development of vascular changes, and increases the generation of fibrosis [Yilmaz et al. 1996, Lautenschlager et al. 1997b, van Dam et al. 2000, Inkinen et al. 2002]. In addition, increased expression of adhesion molecules (ICAM-1, VCAM-1) and growth factors (TGF-β, PDGF, CTGF) was associated with CMV infection [Yilmaz et al. 1996, Kloover et al. 2000, Inkinen et al. 2005]. On the molecular level, several possible mechanisms exist by which CMV may increase the alloresponse and development of chronic changes. CMV is able to infect several cell types in the kidney [Heieren et al. 1988a, Heieren et al. 1988b, Ustinov et al. 1991]. CMV directly or indirectly induces the production of several proinflammatory cytokines, such as IL-1, IL-2, TNF-α [Iwamoto et al. 1990, Geist et al. 1991, Smith et al. 1992], adhesion molecules (ICAM-1, VCAM-1) [van Dorp et al. 1993, Sedmak et al. 1994, Craigen and Grundy 1996], and growth factors (TGF-β, PDGF) [Michelson et al. 1994, Yoo et al. 1996, Srivastava et al. 1999, Zhou et al. 1999], which are all thought to be important in the process of allograft rejection and chronic allograft nephropathy. Chemokines are also important mediators of inflammation in organ transplant pathophysiology. In addition to upregulating the production of several chemokines during infections, the CMV genome encodes chemokine analogues and chemokine receptor analogues, through which the virus is able to further modify immune response and to stimulate cellular responses [Bodaghi et al. 1998, Streblow et al. 1999, Vink et al. 1999].

AIMS OF THE STUDY

The aim of this study was to investigate and clarify the role of cytomegalovirus in the process of chronic allograft nephropathy in clinical kidney transplantation and to study the pathogenetic mechanisms of this suggested association.
The specific objectives were to study:

1. the impact of cytomegalovirus infections and the presence of CMV in the allograft on the development of histopathological changes in six months protocol biopsies (I).

2. the urinary excretion of molecules thought to be important in the development of chronic allograft nephropathy during cytomegalovirus infections (II).

3. the impact of persistent CMV infection in the graft on the expression of cytokines, adhesion molecules and growth factors thought to be important in the development of chronic allograft nephropathy (III).

4. the impact of persistent intragraft CMV infection on long-term kidney allograft function and survival (IV).

MATERIALS AND METHODS

1. Patients (I-IV)

Altogether 1451 adult renal transplantations were performed between the years 1992-2000, and 394 of these patients remained at follow- up at the Helsinki University Hospital Department of Medicine, Division of Nephrology. Primary immunosuppression therapy consisted mainly of cyclosporine, azathioprine and methylprednisolone. No anti-viral prophylaxis for CMV or other herpesviruses was routinely given post-operatively. Trimethoprim-sulfametaxazole prophylaxis against pneumocystis carinii was given routinely during the first six months after transplantation or, if the level of serum creatinine was more than 200μmol/l, pentamidine inhalations were usually used instead. All the patients in this study had a clinical suspicion of CMV infection and samples taken for the diagnosis of CMV.

2. Acute rejections (I-IV)

Acute rejections were diagnosed by fine-needle aspiration biopsy [Hayry and von Willebrand 1981] and/or biopsy histology and by clinical criteria. Biopsy histology was analyzed according to the Banff classification [Solez et al. 1993, Racusen et al. 1999]. Recipients suffering from clinically significant acute rejections received treatment with high-dose intravenous methylprednisolone and, on demand, additionally with OKT3 and/or plasmapheresis.

3. CMV infections

3.1. Detection of CMV infection (I-IV)

The patients in this study were not regularly monitored for CMV and samples for the detection of CMV were obtained only when clinical signs of infection were observed (fever, unexplained increase of serum creatinine, leukopenia, thrombocytopenia, hepatopathy, gastroenteritis, pneumonia). CMV infection was diagnosed by the standard pp65 antigenemia test [The et al. 1995] and rapid shell vial cultures from blood and/or urine [Lautenschlager et al. 1989]. Infections were treated on clinical indications with intravenous ganciclovir. Recipients with CMV infection who received no ganciclovir treatment, demonstrated only occasional low level antigenemia, which subsided during follow-up.

3.2. Demonstration of CMV from the biopsies (I, III-IV)

CMV antigens were detected in kidney allograft biopsies using a monoclonal antibody against CMV specific protein pp65 (Biotest, Dreieich, Germany) and indirect immunoperoxidase staining. The biopsy material was snap-frozen and 3-4 micron thick sections were cut, acetone fixed and stored at -20 C until used. Before staining, the sections were treated with chloroform to avoid nonspecific reactions with endogenous peroxidase. The presence of CMV in kidney allograft biopsies was also demonstrated by DNA hybridization in situ using a biotinylated probe (Enzo Biochem Inc., New York, NY) prepared from a mixture of two clones of CMV sequences in the BamHI site of pBR22 [Lautenschlager et al. 1997a]. To visualize the anatomic structures in the kidney, Mayer's hemalum was used as a counterstain in both the immunoperoxidase stainings and DNA hybridizations in situ.

4. Biopsy technique (I-IV)

Six months biopsies were performed under ultrasound guidance with either Bard Magnum® or Bard Biopty® devices and 18 gauge Biopty-cut® needles®. According to the policy of our clinic, two biopsies were taken: one piece was embedded in Historesin (Leica Instruments GmbH, Heidelberg, FRG) while the other was sectioned for immunofluorescence and the remainder deep frozen at -80C. For light microscopy, serial tissue sections were stained with hematoxilin and eosin, PAS, methenamine silver, and May-Grünwald-Giemsa.

5. Biopsy histology (I-III)

According to the policy of our clinic, a protocol biopsy six months after transplantation was performed for every recipient. The following histological parameters were retrospectively analyzed for the purpose of this study according to the Banff '97 classification [Racusen et al. 1999]: tubulitis and interstitial inflammation as indicators of acute inflammatory response, and glomerular mesangial matrix increase, allograft glomerulopathy, tubular atrophy, interstitial fibrosis and vascular fibrointimal thickening as indicators of chronic allograft damage. Special attention was focused on the analysis of vascular changes and vascular intimal thickening was further specified as arterial or arteriolar. Each vessel identified in the biopsy specimen was scored independently and the average score was recorded.

6. Urine specimens (II)

Urine samples for CMV culture were collected when CMV infection was clinically suspected or when urine samples were collected for other reasons, such as controlling of proteinuria or bacteriuria. Urinary concentrations of TGF-β_1 and soluble ICAM-1

(sICAM-1) were measured by sensitive enzyme immunoassays (EIA) [Honkanen et al. 1997] (Bender MedSystems, Vienna, Austria for sICAM-1), and radio immunoassay (RIA) was used to measure urinary excretion of TNF-α [Teppo and Maury 1987]. Pharmacy albumin RIA was used to measure urinary albumin. The excretions of the analyzed molecules were related to concomitant urinary creatinine content (millimoles).

7. Immunohistochemistry (III)

The expression of TGF-β_1, PDGF-AA, PDGF-BB, ICAM-1, ELAM, VCAM-1, IL-2 receptor, CD4, CD8, and MHC class II antigen were demonstrated in frozen sections (3-5 μm thick) of the kidney allograft biopsies by indirect immunoperoxidase staining. Monoclonal antibodies were used for ICAM-1, VCAM-1, ELAM-1 (R&D Systems, Abignon, UK), IL-2R (Becton Dickinson Immunocytometry Systems, San Jose, USA) CD4, CD8 (Southern Biotechnology Associates, Inc, Birmingham, USA), and MHC class II (DAKO A/S, Clostrup, Denmark) and polyclonal antibodies for TGF-β_1, PDGF-AA, and PDGF-BB (R&D Systems, Abignon, UK). Expression was scored semiquantitatively on a scale from 0 to 3 or by counting positive leukocyte cells per high power visual field (in the case of IL-2R, CD4, and CD8). All biopsies were scored by two researchers. Expression was scored separately in tubuli, glomeruli, arterial and peritubular capillary endothelial cells and in vascular smooth muscle cells.

8. Clinical variables (I-IV)

Kidney allograft function was measured as the level of serum creatinine (I-IV) and creatinine clearance (ml/s/1.73m^2) was measured from 24 hour urine collection (I) or was calculated from serum creatinine, age, gender, and body weight using the Cockcroft-Gault- formula [Cockcroft and Gault 1976] (IV). In addition, the amount of protein in 24h urine collection was determined (I).
The following baseline data at the time of transplantation were obtained from patient files: recipient and donor age and gender, primary renal disease, number of HLA-A, B, and DR mismatches, cold ischemia time, onset of graft functions (days post-transplantation). Systolic and diastolic blood pressure and blood trough level of cyclosporine A, glycosylated haemoglobin, and total serum cholesterol values were obtained from the patient files at six months (I, III), at the time of the urine sample (II), or annually after transplantation (IV).

9. Statistical methods (I-IV)

All data are expressed as mean±1 standard deviation, unless otherwise indicated. Differences between two groups in the distribution of continuous variables were analyzed with the nonparametric Mann-Whitney U-test and differences between the three groups

were analyzed with the nonparametric Kruskal-Wallis one-way analysis. Nonparametric tests were chosen because all distributions were not normal and because of small sample size (I-IV). Graft survival probabilities were estimated by the Kaplan-Meier method, and differences between two or more groups were analyzed by the log rank test. Univariate Cox regression analysis was used to calculate relative risks (RR) and 95 % confidence intervals (CI) of graft failure. Graft survival was analyzed both uncensored for death, i.e. death with a functioning graft was considered as an event and censored for death, i.e. deaths with functioning grafts were censored. For logistic regression analyses, creatinine clearance values were converted to binary and used as outcome variables. The median was used as a cut point to obtain two categories. For multivariate analyses, the three most significant variables in univariate analysis were chosen (IV).

The calculations were performed with StatView (I) (version 512+, Brainpower Inc., Calabasa, CA) and SPSS statistical software (II-IV) (version 12.0.1., SPSS Inc, Chicago, IL). Two tailed P-values lower than 0.05 were considered statistically significant.

RESULTS

From the patients who remained at follow-up at Helsinki University Hospital between the years 1992-2000, 172 patients had a suspicion of CMV infection and CMV was diagnosed in 82 recipients. The control group comprised 23 patients with no evidence of CMV infection despite several samples taken at different time points after transplantation. Patients included in the study are described in figure 3.

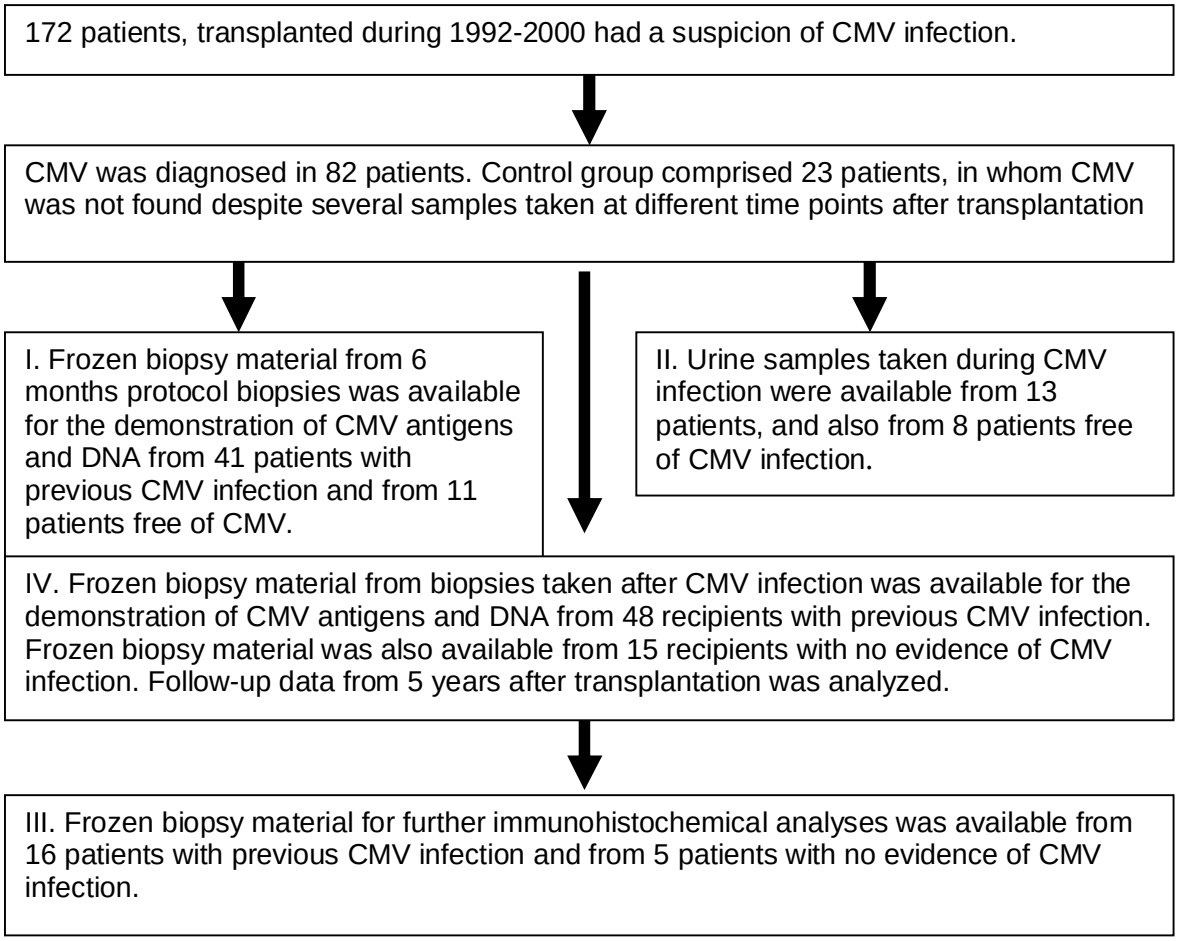

Figure 3. *Description of the patients included in the studies I-IV.*

1. Intragraft CMV and histopathological changes (I)

Histopathological changes were scored from 6-month protocol biopsy specimens from 52 renal allograft recipients, from whom frozen biopsy material from the protocol biopsies was available for the demonstration of CMV pp65 antigens and DNA hybridization in situ. All patients had a clinical suspicion of CMV infection and had samples taken for the detection of CMV infection. In 18 of the 41 patients with CMV infection, CMV antigens or DNA were found in the 6-month protocol biopsy specimens. Of these 18 patients with intragraft CMV, 11 suffered from several episodes of CMV infection. The control group comprised 11 patients, in whom CMV was not diagnosed despite several samples taken at different time points after transplantation. No CMV DNA or proteins were found in the biopsies of these 11 patients.

No differences in the histopathological parameters analyzed were recorded in patients with previous CMV infection (N=41) compared to patients free of CMV infection (N=11). However, in the biopsies of patients with a positive CMV finding by immunohistochemistry or DNA in situ hybridization (N=18), the intensity of fibrous intimal thickening in the small arterioles was significantly increased compared to recipients with no history of CMV infection ($p<0.01$). No differences in the other histopathological parameters were found. Acute rejection developed in 22 of the 41 patients with CMV infection and in 6 of 11 recipients free of CMV infection (p=non significant, ns). No differences were found in the severity or the number of acute rejection episode between the recipients with CMV infection and no CMV infection. No differences were found in the pretransplant CMV antibody status between the patients with intragraft CMV and patients with no previous CMV.

When the study patients were further analyzed with regard to acute rejections, no differences in vascular or other histopathological changes were found in the subgroup of patients without previous acute rejection episodes, despite their CMV status. In patients free of CMV infection, previous acute rejection episodes were not associated with increased vascular or other histopathological changes. However, in recipients with acute rejections as well as a positive CMV finding in the graft, the intima of the small arterioles was significantly thickened as compared to recipients without CMV infection ($p<0.05$) (Figure 4). The groups of patients with previous CMV infection, intragraft CMV, or no previous CMV, did not differ in renal function parameters.

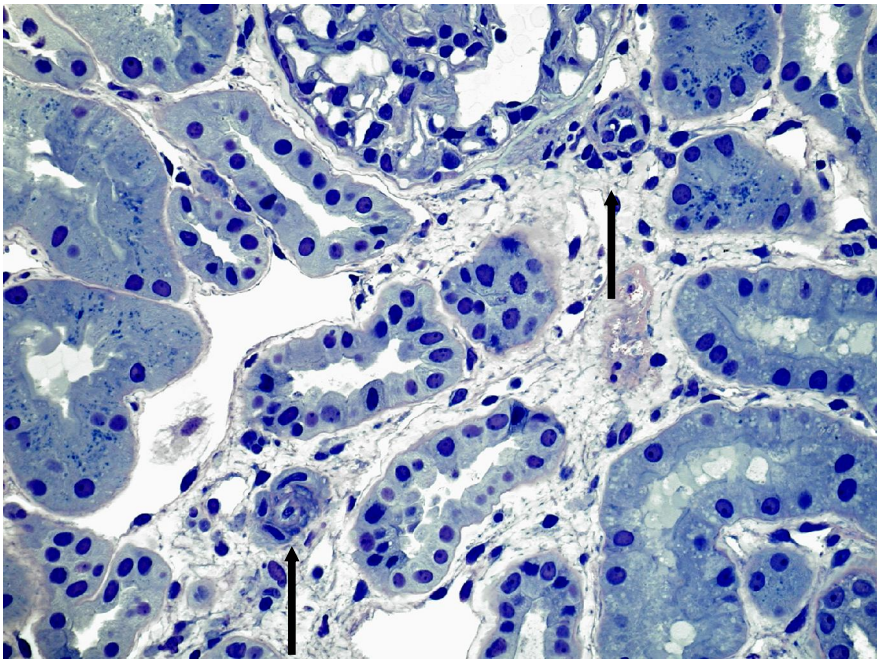

Figure 4. *Small occluded arterioles (arrows) in a biopsy of a patient with both a positive CMV finding in the graft and a previous history of acute rejection.*

2. Urinary excretion of soluble molecules during CMV infection (II)

Urine samples from 46 renal transplant recipients were available for this study. Samples were taken due to suspicion of CMV infection, acute rejection, or controlling of proteinuria or bacteriuria. Urine samples taken from patients with previous CMV infection (n= 22) or CMV infection after the urine sample (n=3), were excluded from the analysis. Thirteen patients had CMV infection at the time of the urine sample (CMV group), as demonstrated by a positive antigenemia test in 10 patients and in 3 patients by a positive culture from urine. The 8 patients in whom CMV infection was not diagnosed at any time point were used as controls. The two study groups did not differ in any of the demographic factors analyzed.

The urinary excretion of TGF-β_1 was significantly higher in the urine samples taken during a CMV infection CMV (N=13) compared to controls with no evidence of previous or ongoing CMV infection (N=8) ($p<0.001$) (Table 2). No differences were recorded between the CMV group and the controls in the excretions of other cytokines analyzed or of albumin. At the time of the urine sample, 3 patients from the CMV group and 3 patients from the control group, suffered from acute rejection. Ongoing acute rejections were not associated with increased excretion of the molecules analyzed. None of the pre or perioperative factors, time of the urine collection after transplantation, serum

creatinine, blood trough levels of cyclosporine, previous acute rejections, nor the amount of proteinuria, had any correlation with urinary excretion of any of the molecules analyzed.

In the 13 patients with a positive history of CMV infection and increased urinary levels of TGF-β_1, a significantly higher degree of interstitial fibrosis was recorded in the six months protocol biopsies ($P<0.01$). Tubular atrophy was also somewhat increased in patients with previous CMV, although not statistically significantly. No differences existed in the other histopathological parameters analyzed. Previous acute rejection episodes were not associated with increased histopathological changes in any of the parameters analyzed. Urinary excretion of TGF-β_1 correlated positively, although not statistically significantly, with the degree of interstitial fibrosis ($R=0.43$, $p=0.08$).

Protein / Creatinine	**Control group (n=8)**	**CMV group (n=13)**
S-ICAM- 1 (ng/mmol)	144.1 ± 97.2	275.0 ± 363.2
TNF- α (ng/mmol)	0.49 ± 0.35	0.33 ± 0.32
TGF- β_1 (ng/mmol)	13.3 ± 6.7	51.1 ± 28.0*

Table 2. *The impact of CMV infections on the urinary excretion of S-ICAM –1, TNF- α, and TGF- β_1. All data expressed as mean±SD. *p < 0.001.*

3. Persistent CMV in the allograft (III-IV)

Frozen biopsy material from biopsies taken after CMV infection were available from 48/82 patients for the demonstration of CMV antigens or DNA in the biopsies. Frozen biopsy material was available for the demonstration of CMV also from 15 patients with no evidence of previous CMV infection, despite several samples taken at different time points after transplantation. These 63 recipients were further analyzed. In 17/48 patients with a positive history of CMV infection, CMV persisted in the biopsy, as CMV antigens or DNA were demonstrated in the allograft biopsies 2-12 months after the last positive finding in blood or urine (persistent CMV group). In 31/48 recipients with previous CMV infection, and also in the 15 patients free of CMV, no CMV DNA or antigens were found in the biopsies (non-persistent CMV and no CMV groups respectively). No differences were found in the number or severity of acute rejection episodes or in the pretransplant CMV antibody status between patients with persistent intragraft CMV compared to the other groups. Of the biopsies analyzed, 12/17 were protocol biopsies in the persistent CMV group, 22/31 in the non-persistent CMV group, and 9/15 in the no CMV group

(p=ns). No significant differences between the study groups were recorded in the time of the biopsy sample after transplantation.

3.1. Immunohistochemical analyses (III)

Frozen biopsy material for the immunohistochemical stainings was available for further analyses from 11 patients with persistent intragraft CMV, from 5 patients with non-persistent CMV, and from 5 patients free of CMV infection. In the immunohistochemical analyses, the expression of TGF-β_1 was significantly increased in arterial endothelial cells in patients with persistent intragraft CMV, compared to patients with no CMV (p=0.036). Similarly, the expression of PDGF-AA was significantly increased in tubuli (p=0.040) and the expression of ICAM-1 in peritubular capillary endothelium (p=0.003) in patients with persistent CMV in the graft, compared to patients free of CMV (table 3). No differences were recorded in the expression of the other molecules analyzed between the groups. Previous acute rejection episodes were not associated with increased expression of any of the molecules analyzed in the immunohistochemical stainings.

In the patients with persistent CMV and increased intragraft expression of TGF-β_1, PDGF-AA and ICAM-1, analysis of the 6- month protocol biopsy histology showed a higher degree of arterial intimal thickening compared to patients who were free of CMV infection (P=0.02). No differences were found in the other histopathological parameters analyzed.

TGF-β_1	Tubuli	Capillary endothelium	Arterial Endothelium
Controls (n=5)	0.5 ± 1	0 ± 0	0 ± 0
Persistent CMV (n=11)	1.3 ± 1	0.5 ± 0.8	1.0 ± 1.2*
PDGF-AA			
Controls (n=5)	0.5 ± 0.6	0.8 ± 1.0	0.5 ± 0.6
Persistent CMV (n=11)	1.6 ± 0.9**	1.4 ± 1.0	0.9 ± 0.8
ICAM-1			
Controls (n=5)	0 ± 0	1.6 ± 0.5	0.5 ± 0.6
Persistent CMV (n=11)	0.3 ± 0.9	2.8 ± 0.4***	0.3 ± 0.9

Table 3. *The impact of persistent CMV infection on the expression of TGF-β_1, PDGF-AA and ICAM-1. All data expressed as mean±SD. *p=0.036, **p=0.040, ***p=0.003*

3.2. Long- term graft function and survival (IV)

Graft survival (uncensored for death) in patients with persistent intragraft CMV was significantly reduced compared to patients with non-persistent CMV or no CMV (P=0.049). A similar trend was seen in death-censored graft survival (P=0.11, NS). When patients with persistent CMV in the graft were compared to patients with no evidence of intragraft CMV (i.e. non-persistent CMV and no CMV groups combined), persistent CMV was associated with significantly reduced graft survival both uncensored for death and death-censored (P=0.020 and P=0.041 respectively) (Figure 5).

In Cox regression analyses including several pre or postoperative risk factors for graft loss, persistent intragraft CMV infection was the only significant risk factor for graft loss, uncensored for death (relative risk 3.5, 95% confidence interval 1.1–10.9, P=0.030). The only significant risk factor for death-censored graft survival was the number of HLA-mismatches (RR 5.0, P=0.028), although borderline increased risk was also associated with persistent intragraft CMV (RR 5.3, P=0.068).

Kidney allograft function, as measured by estimated creatinine clearance, was significantly reduced in patients with persistent intragraft CMV compared to non-persistent and no CMV groups at one and two years (at one year 0.89±0.32 vs. 1.16±0.31 and 1.05±0.26 ml/s/1.73m^2 respectively, P=0.018 and at two years 0.84±0.32 vs. 1.12±0.31 and 0.99±0.35 ml/s/1.73m^2, P=0.012). In univariate logistic regression analyses, persistent CMV and higher donor age showed to be the only significant risk factors for lower clearance one year after transplantation (OR 7.69 for CMV, P = 0.004, OR 1.06 for one year increase in donor age, P=0.014). Similarly, in multivariate logistic regression analysis, persistent intragraft CMV and higher donor age appeared as independent risk factors for lower clearance one year after transplantation (OR 4.4 for CMV, P=0.027, and OR 1.04 for one year increase in donor age, P=0.049). At two years, persistent CMV was the only significant risk factor for lower clearance in univariate analysis (OR 4.3, P=0.026) and the only significant independent risk factor for lower clearance two years after transplantation in multivariate analysis (OR 4.9, P=0.020). No differences existed in the creatinine clearance values at three to five years after transplantation between the persistent CMV group and the other groups.

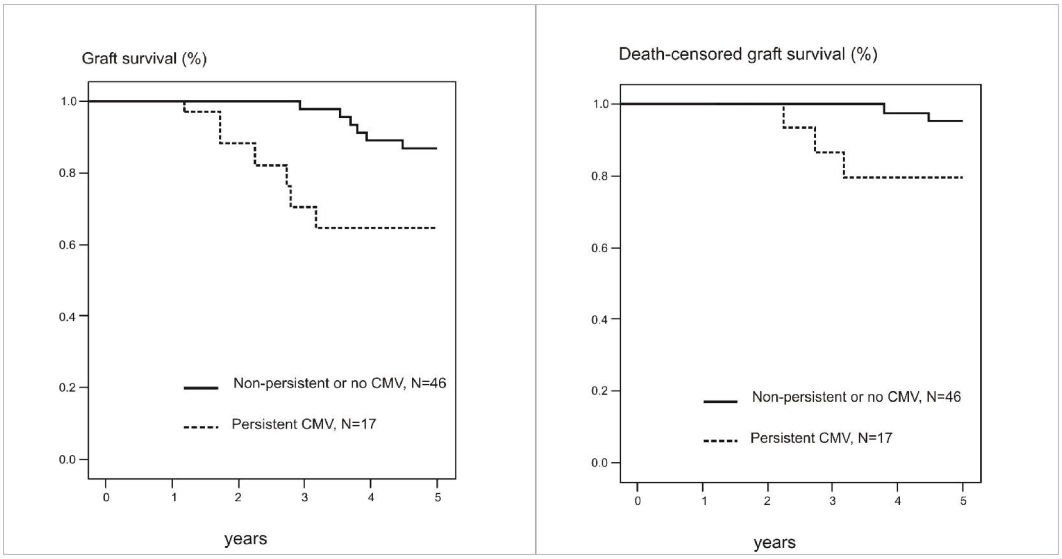

Figure 5. *Reduced graft survival (uncensored for death) (P=0.020) and death- censored graft survival (P=0.041) in patients with persistent CMV compared to patients with non-persistent or no CMV.*

DISCUSSION

Cytomegalovirus is an important pathogen infecting organ transplant recipients. In addition to the complications associated with acute infection, CMV is a suggested risk factor for chronic rejection after solid organ transplantation. The best clinical evidence supporting this hypothesis derives from heart transplantation [Grattan et al. 1989, Loebe et al. 1990, Koskinen et al. 1993, Valantine et al. 1999]. Some studies support a role for CMV in the development of vanishing bile duct syndrome (chronic rejection) after liver transplantation [O'Grady et al. 1988, Arnold et al. 1992, Lautenschlager et al. 1997a, Evans et al. 1999], and obliterative bronchiolitis after lung transplantation [Kroshus et al. 1997]. The role of CMV in chronic allograft nephropathy after transplantation of the kidney is more controversial. Most of the data on the association of CMV and CAN are experimental; clinical evidence is still scarce and some studies have found no association between CMV and CAN (see table 1). The aim of this study was to investigate the possible association of CMV and CAN in humans in detail and also to study the mechanisms involved.

1. CMV and renal allograft histopathology

Although CMV infections after transplantation have been extensively studied, surprisingly little data exist on the association of CMV infection with CAN, as defined by histopathological changes in allograft. Most of the studies have only associated CMV with clinical or laboratory parameters, and no systematic histological analyses have been presented. In the present study, clinical CMV infection was not associated with any histopathological changes in the graft. However, we found CMV antigens or DNA in glomerular, tubular and vascular structures in 18/41 of the 6-month protocol biopsies of kidney allograft recipients after CMV infection, and this intragraft CMV infection seemed to be associated with increased vasculopathic changes in small arterioles. Further analysis revealed that intragraft CMV infection was associated with increased vasculopathic changes in small arterioles only together with a previous history of acute rejection episodes (I). Neither acute rejection nor CMV in the graft alone was associated with any histopathological changes.

CMV is able to infect several cell types in the kidney in cell cultures, including glomerular, tubular and endothelial cells [Heieren et al. 1988a, Heieren et al. 1988b, Ustinov et al. 1991]. Also in clinical kidney transplantation, the virus has been detected in the allograft [Ulrich et al. 1986, Andersen et al. 1990, Lardelli et al. 1994, Holma et al. 2000, Liapis et al. 2003] and evidence shows that CMV is able to persist in the allograft for months after viremia and active infection (III, IV) [Holma et al. 2000]. This

persistence has been associated with chronic changes in the graft [Holma et al. 2000], (III). Persistent viral infection in the graft may be able to stimulate inflammatory cells both indirectly and also by direct effects of CMV on the production of several growth factors and cytokines (III). This continuous injury in the graft, together with an additional injury caused by a previous acute rejection episode, may have caused persistent low-level inflammation, leading to growth factor stimulation and the development of vascular changes, as seen in our study (I, III).

2. CMV and the mechanisms of CAN

The studies investigating the possible association of CMV and CAN are mainly based on animal models. An association of rat CMV infection and chronic renal allograft rejection has been described in several models [Yilmaz et al. 1996, Lautenschlager et al. 1997b, van Dam et al. 2000]. In the rat, CMV infection increased inflammation, accelerated the development of chronic vascular and fibrotic changes [Yilmaz et al. 1996, Lautenschlager et al. 1997b, Inkinen et al. 2002] and was associated with increased expression of several molecules, such as ICAM-1, VCAM-1, TGF-β, PDGF, and CTGF, which are all thought to be important in the development of chronic rejection [Yilmaz et al. 1996, Kloover et al. 2000, Inkinen et al. 2005]. Clinical studies of the mechanisms behind the suggested association of CMV and CAN mainly involve analyses of proinflammatory cytokine levels [Kern et al. 1996, Nordoy et al. 2000a, Nordoy et al. 2000b, Tong et al. 2001].

2.1. CMV and proinflammatory cytokines and adhesion molecules

CMV modulates the immune response through several proinflammatory cytokines, and reactivation of CMV from latency may be stimulated by cytokines produced during the alloimmune response, such as TNF-α, IL-1, and IFN-γ [Soderberg-Naucler and Nelson 1999, Hummel and Abecassis 2002].The key cytokine in the association of CMV and the rejection cascade is suggested to be tumor necrosis factor –α (TNF-α) [Smith et al. 1992, Docke et al. 1994, Fietze et al. 1994]. CMV directly upregulates the production of TNF-α in monocytes and macrophages [Smith et al. 1992] and on the other hand, TNF-α stimulates the activity of CMV via the direct actions of transcription factor NF-κB on the viral genome [Fietze et al. 1994, Prosch et al. 1995]. This bidirectional mechanism may enhance alloresponse and injury to the graft. In addition, CMV may directly stimulate the production of IL-1 in mononuclear cells [Iwamoto et al. 1990]. These cytokines IL-1 and TNF-α in turn stimulate the synthesis and release of growth factors TGF-β and PDGF [Phan et al. 1992, Pintavorn and Ballermann 1997, Funayama et al. 1998], which are associated with the pathobiology of CAN. CMV also directly stimulates the production of

IL-2 and IL-2 receptor, which are important molecules in the alloimmune response [Geist et al. 1991].

Proinflammatory cytokines IL-1 and TNF-α also stimulate the expression of adhesion molecules ICAM-1 and VCAM-1, which are key adhesion molecules in the alloimmune response and have also been associated with chronic changes. In experimental studies, CMV is able to directly stimulate the expression of ICAM-1 both in vitro and in vivo [van Dorp et al. 1993, Craigen and Grundy 1996, Burns et al. 1999, Kloover et al. 2000]. Persistent intragraft CMV infection was, in our study, associated with increased expression of ICAM-1 in the peritubular capillaries (III). As ICAM-1 is constitutively expressed in transplanted kidneys with normal histology [Solez et al. 1997], some extent of ICAM-1 was seen in all the biopsies analyzed. However, the expression was significantly more intense in recipients with persistent intragraft CMV infection.

2.2. CMV and increased expression of TGF-β

TGF-β is thought to be one of the key fibrogenic molecules in the development of CAN, and it also has some vasculopathic potential, mostly mediated by increased expression of PDGF. In vitro, CMV immediate early genes directly induce the secretion of TGF-β in endothelial cells [Yoo et al. 1996]. In addition, proinflammatory cytokines IL-1 and TNF-α, which are directly stimulated by CMV, increase the production of TGF-β [Phan et al. 1992, Pintavorn and Ballermann 1997].

In an experimental rat model of chronic renal allograft rejection, rat CMV infection increased the expression of TGF-β and CTGF in the graft and increased collagen synthesis and the degree of interstitial fibrosis [Inkinen et al. 2002, Inkinen et al. 2005]. CTGF is an important profibrotic growth factor in renal fibrosis [Ito et al. 1998], and most of the profibrotic effects of TGF-β are thought to be mediated by increased secretion of CTGF caused by TGF-β [Grotendorst 1997]. We found increased expression of TGF-β in the allograft associated with persistent intragraft CMV infection (III), and increased vasculopathic changes were recorded in recipients with persistent CMV infection in the graft.

Increased urinary excretion of TGF-β has been described in several fibrotic kidney diseases. Although most of the antibodies used to detect TGF-β are unable to differentiate between active and latent form of TGF-β, increased urinary levels of TGF-β are thought to reflect increased production of the molecule and have been associated with histopathological changes and prognosis [Honkanen et al. 1997, Korpinen et al. 2000], also in renal transplant recipients [Boratynska 1999, Teppo et al. 2004, Rogier et al. 2005]. We found significantly increased urinary levels of TGF-β during CMV infection

in kidney allograft recipients (II). This novel finding was associated with histologic evidence of increased degree of interstitial fibrosis, indicating the clinical significance of this observed increase in the production of TGF-β. We hypothesize that CMV infection may enhance the expression of TNF-α, which in turn increases the production of TGF-β, also directly stimulated by CMV. TGF-β is a profibrotic molecule and also mediates some of its profibrotic effects by stimulating the expression of CTGF, leading to increased collagen synthesis and fibrosis, as has been earlier observed in an experimental model [Krogerus et al. 2003, Inkinen et al. 2005].

2.3. CMV and increased expression of PDGF

PDGF is an important stimulating growth factor for mesenchymal cells, and increased expression of PDGF has also been associated with the development of chronic allograft nephropathy [Fellstrom et al. 1989, Floege et al. 1998]. According to some studies PDGF-BB, or actions mediated by PDGF receptor β, is thought to be the most important isoform in several pathogenetic states, such as atherosclerosis [Betsholtz and Raines 1997]. However, in chronic renal vascular rejection, PDGF-AA is expressed by intimal and medial smooth muscle cells in arteries, whereas PDGF-BB is mostly expressed in infiltrating monocytes [Floege et al. 1998]. In our study, increased expression of PDGF-AA was observed in tubuli and also slightly in arterial endothelium of patients with persistent intragraft CMV, whereas the expression of PDGF-BB was scarce and seen mostly in the infiltrating inflammatory cells (III). In vitro studies show that CMV is able to directly induce the production and secretion of PDGF in endothelial and smooth muscle cells [Srivastava et al. 1999, Zhou et al. 1999]. Moreover, in an experimental rat model of chronic allograft rejection, CMV infection was associated with increased expression of PDGF [Inkinen et al. 2005]. These findings support our hypothesis of CMV being able to stimulate the expression of PDGF and thus accelerate the progression of CAN, recorded in our study as increased vascular changes (I, III).

3. CMV and long-term prognosis

In biopsies taken after CMV infection, CMV persisted in 17/48 of kidney grafts, i.e. CMV antigens, DNA, or both, were found in the biopsy more than 2 months after the last positive finding in blood or urine. Similar persistence of CMV in the graft has been observed after liver transplantation and also in the kidney [Lautenschlager et al. 1997a, Holma et al. 2000], but the nature and relevance of this persistence in the kidney is poorly described. In our material, in addition to the histopathological changes, this persistence in the kidney allograft was associated with significantly reduced graft function and survival (IV).

Previous studies of the association of CMV and CAN have only investigated the role of CMV infection or disease as detected by viremia, and intragraft CMV status has not been detected. We hypothesize that it is in particular the long-term injury caused by a persistent CMV infection in the graft, which is associated with the development of chronic allograft nephropathy. The intragraft CMV infection observed in our study could explain the controversial results of previous CMV studies (table 1), since we found no association between "transient" CMV infection and graft function, survival, or histopathological changes. All significant differences were associated with CMV detected in the allograft. Reduced kidney function was recorded only in recipients with persistent intragraft infection (IV) but not in recipients with intragraft CMV infection only in the 6-month protocol biopsies (I).

The incidence of intragraft CMV infection has not been studied prospectively, and this retrospective study from an era before the current antiviral prophylaxis and preemptive protocols, including only patients with a suspicion of CMV infection, is not sufficient to answer the question of how common this intragraft infection with CMV is. Similarly, the factors that might favour the persistence of CMV in the graft are largely unknown. In our study, slightly increased donor age and HLA mismatch was seen in the baseline characteristics of recipients with persistent CMV infection in the graft. Both of these factors are able to increase the immunogenicity of the allograft; increased alloreactivity may trigger the virus and favour the persistence of CMV.

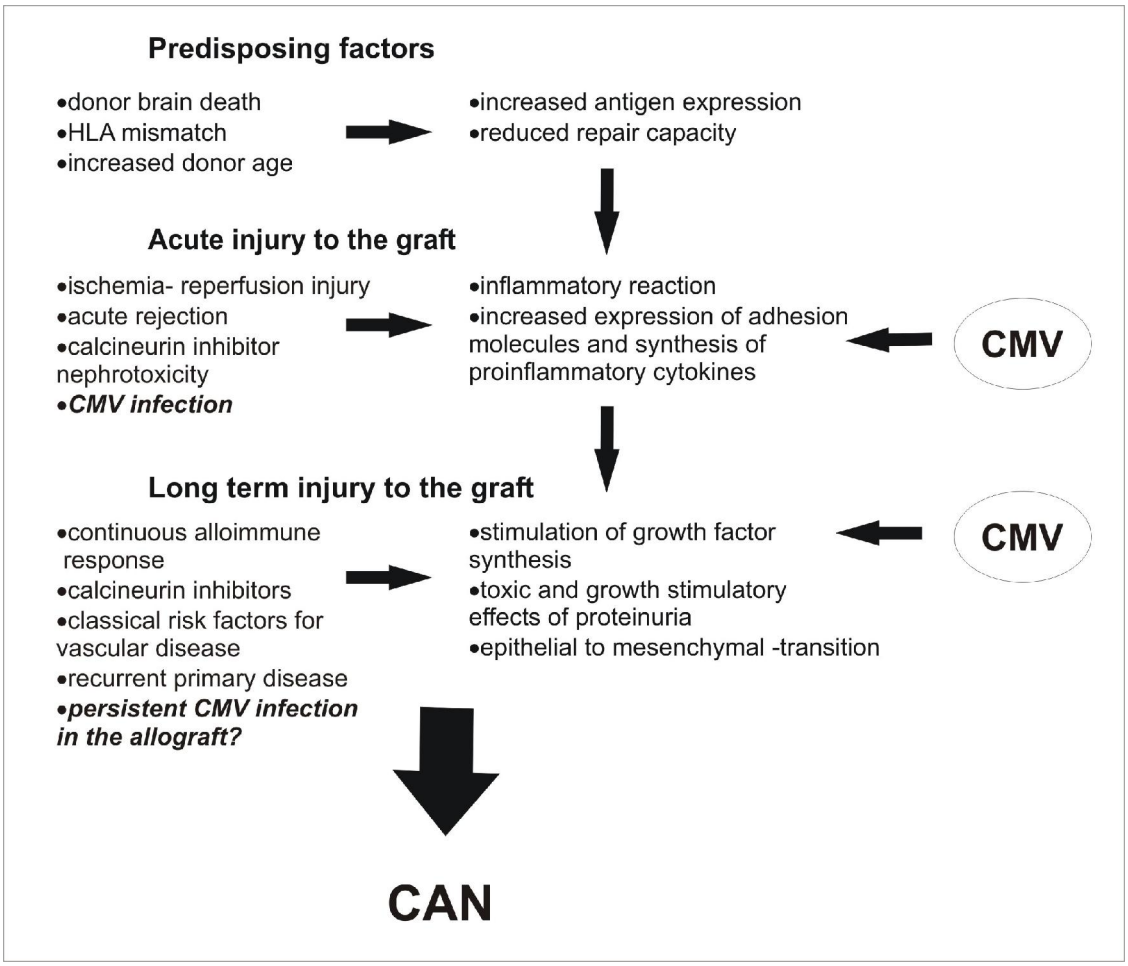

Figure 6. Schematic representation of the role of CMV in the pathogenesis of CAN.

4. Conclusions

The findings of our study suggest that persistent CMV infection in the kidney allograft is detrimental to long-term kidney allograft function and survival. In recipients with both intragraft CMV infection and previous acute rejection episodes, increased vasculopathic changes were recorded in small arterioles in 6-month protocol biopsies. Furthermore, persistent CMV infection was associated with increased expression of adhesion molecule ICAM-1 and growth factors TGF-β and PDGF, which are all thought to be important molecules in the process of chronic allograft nephropathy. Similarly, urinary excretion of TGF-β was increased during CMV infections. In five years follow-up, persistent CMV infection was associated with reduced graft function and survival.

Based on these results, we hypothesize that persistent CMV infection in the kidney allograft, especially in combination with other injuries to the graft, is able to cause continuous injury to the allograft by increasing inflammatory and growth factor response, resulting in arteriosclerotic changes and interstitial fibrosis, and finally manifesting as reduced allograft function and survival. These novel findings of persistent intragraft infection may explain the previous controversial results of the impact of CMV infection on kidney allograft survival, since the effect persistence of CMV has not been demonstrated in earlier reports. These results strongly suggest that effective prevention, diagnosis, and treatment of CMV infections may be a major factor in improving the long-term prognosis of kidney transplantation.

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Ilkka Helanterä

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